Clean version of the replacement paragraphs

Specification, paragraph on page 31, lines 16 to 20:

B1

The present invention is also illustrated by the following examples. The list of figures concerning these examples is given hereafter. Figures 1 to 7 are described in example 1, figures 8 to 10 in example 2, figures 11 and 12 in example 3 and figure 13 in example 6.

Specification, paragraph on page 31, lines 22 and 23:

B2

Figure 1 : Growth of the strains M5 and M5 *fil1* = PVD1150 on YPD medium at 30°C and under stirring (180 rpm) according to test T3.

Specification, paragraph on page 31, lines 24 to 26:

B3

Figure 2 : cAMP response (Arbitrary Units) of the strains PVD1150 and M5 after addition of glucose (100 mM) to cells cultured on glycerol medium until obtaining of stationary phase.

Specification, paragraph on page 31, lines 27 to 29:

B4

Figure 3 : Monitoring of the degradation of trehalose (Arbitrary Units) in the strains PVD1050, PVD1150 and their respective controls M5 *hxk2Δ* and M5, after an induction by glucose (200 mM) on cells in stationary phase.

Specification, paragraph on page 31, lines 30 and 31:

B5

Figure 4 : Survival rate of the strains HL8.16 *leu2* and HL816 *fil300* after a thermic shock of 30 minutes at 52°C according to test T1.

Specification, paragraph on page 32, lines 1 and 2:

B6

Figure 5 : Survival rate of the strains HL8.16 *leu2* and HL816 *fil300* after a freezing of 12 days at -20°C according to test T2.

Specification, paragraph on page 32, lines 3 to 5:

B7

Figure 6 : Trehalose content (Arbitrary Units) in the strains HL8.16 *leu2* and HL816 *fil300*. The strains are grown until obtaining of stationary phase, and then glucose (100 mM) is added at t=0.

Specification, paragraph on page 32, lines 6 to 9:

B8

Figure 7 : cAMP response (Arbitrary Units) after induction by glucose (100 mM) in the strains HL8.16 *leu2* and HL816 *fil300* cultured until obtaining of stationary phase. The initial adding of 3 mM of glucose allows to avoid the cAMP response related to intracellular acidification.

Specification, paragraph on page 32, lines 10 to 12:

B9

Figure 8 : Monitoring of the degradation of trehalose (Arbitrary Units) after an induction by glucose at t = 0 (on cells of strains S47, AT25, AT26, AT28, AT31 in stationary phase).

Specification, paragraph on page 32, lines 13 to 15:

B10

Figure 9 and figure 10 : Monitoring of the cAMP response (Arbitrary Units) after an induction (at t = 0) by glucose on cells in exponential growth phase on maltose. a) control S47 and mutant AT25 ; b) control S47 and mutants AT26, AT28 and AT31.

Specification, paragraph on page 32, lines 16 and 17:

B11

Figure 11 : Gap-filling strategy (filling of missing DNA) used for isolating the gene carrying the *fil1* mutation in the strain PVD1150.

Specification, paragraph on page 32, line 18:

B12

Figure 12 : Physical map of the vector pUC18-CYR1mut -URA3 [Sn].

Specification, paragraph on page 32, lines 19 and 20:

B13

Figure 13 : Stability to freezing measured by the ratio between the gassing power after 1 day of conservation at -20°C and n days of storage at -20°C.

Specification, paragraph on page 34, lines 9 to 15:

B14

In a first step, the growth of the strain PVD1150 on glucose has been compared with that of the control M5 in order to study the possible influence of the *fil1* mutation on the growth. The strains have been cultured on YPD medium according to the conditions of test T3. The results (figure 1) clearly show that the *fil1* mutation very slightly affects the growth of the strain M5. These same strains are then cultivated on molasses agar according to the conditions of test T5. An identical growth yield has been obtained in the case of the two strains (table 1-B).

Specification, paragraph on page 35, lines 7 to 19:

B15

In the case of the two strains presenting the *fil1* mutation (PVD1050 and PVD1150) the levels of cAMP and of trehalose were examined with respect to their respective controls (M5 *hxk2Δ* and M5). The cAMP level has been measured on the strains PVD1150 and M5, according the method disclosed by Thévelein et coll., 1987, J. Gen. Microbiol., 133, pp.2197-2205. The cAMP level has thus been determined after induction of its synthesis by addition of glucose (100 mM) on cells which have reached the stationary phase after growth on glycerol. An attenuated cAMP signal has been shown in the mutant PVD1150 (figure 2). Trehalose has also been determined on the strains PVD1150, PVD1050, M5 and M5 *hxk2Δ*, according to the conditions described by Neves et coll., 1991, FEBS Lett., 283, pp.19-22. In the case of strains

B15 cont.

presenting the *fil1* mutation, the mobilization of the trehalose (after induction by glucose on cells in stationary phase) is far less rapid than in the control strains (figure 3).

Specification, paragraph on page 37, lines 17 to 25:

B16

A significant improvement of the heat resistance of the strain has been confirmed by test T1, either in stationary phase or in active fermentation phase (figure 4) . Furthermore, mutation *fil300* induced a resistance against other stresses : strain HL816 *fil300* presents, under conditions of test T2, a survival rate of more than 50% after freezing during 12 days at -20°C of prefermented cells during 90 minutes at 30°C while the survival rate of the control is in the same conditions lower than 11% (figure 5). The strain HL816 *fil300* presents consequently a much better resistance against freezing than the control strain, whether the cells are in stationary phase or in active fermentation phase.

Specification, paragraph from page 37, line 26, to page 38, line 2:

B17

In order to better characterize the phenotype linked to the *fil300* mutation, the levels of trehalose and cAMP have been determined on cells in stationary phase which were subjected to an induction by glucose. The control strain is the starting strain HL8.16 *leu2*. During stationary phase (i.e. 0 minute of fermentation in figure 6), the trehalose level is 3 to 4 times higher in mutant *fil300* than in control HL8.16 *leu2*. During exponential phase, the degradation rate of trehalose in the mutant HL8.16 *fil300* strain is reduced, with respect to that of the control (figure 6). In mutant HL816 *fil300* a cAMP response reduced by close to 50% after an induction of the synthesis by glucose on cells in stationary phase has also been observed (figure7).

Specification, paragraph on page 43, lines 1 to 21:

B18

In order to check whether the mutations carried by the strains AT25, AT26, AT28 and AT31 also affect the Ras-cAMP pathway as in the case of the fil strains described in example 1, the levels of cAMP and of trehalose in these different strains

have been measured. The measurements of trehalose have been carried out on cells cultured until the stationary phase and then subjected to an induction of its mobilization or degradation by addition of glucose (100 mM). The initial level of trehalose, which corresponds to the level of trehalose in stationary phase, is higher for all the mutants than for the control (t=0, figure 8). In the presence of glucose, the degradation of trehalose is rather quick for each of the said strains (mutants and control) as after 20 minutes and more, a low and almost identical level of trehalose is obtained for all of these strains. However, the stress imposed within the frame of test T2 is realized after an incubation of 30 minutes in the presence of glucose. This confirms, here again, that trehalose does not allow an explanation of the better performances of these fil mutants. As far as cAMP is concerned, the measurements were carried out on cells which were in exponential growth phase on maltose which have been subjected to an induction by glucose (100 mM). In these experiments, there is no significant reduction of the cAMP signal in the mutants with respect to the control strains S47 (figure 9 and figure 10). Consequently, it seems that the cryoresistant mutants AT25, AT26, AT28 and AT31, which had been isolated following successive cycles of freezing/thawing and which have the fil phenotype, carry mutations which affect other targets than the Ras-cAMP pathway.

B18 cont.

Specification, paragraph on page 44, lines 1 to 18:

It has been determined that the *fil1* mutation is located close to the centromere of chromosome X. The strain PVD1150 has consequently been complemented with each of the genes located in that region, according to the following general strategy :

- transformation of the strain PVD1150 with centromeric plasmids carrying each one of the genes located at less than 25 kb from the centromere of chromosome X,
- search for the transformant clones having lost the phenotype of thermoresistance (= heat-resistance),
- isolation of the gene carrying the mutation from the mutant PVD1150 by way of the technique of " gap-filling " hereafter defined and illustrated by figure 11; retransformation of the strain PVD1150 with the mutated gene thus isolated in order to verify that it is not a suppressor gene,

B19

B19 cont.

- sequencing of the mutated gene originating from PVD1150 and of its wild type allele originating from M5 in order to identify and to locate the mutation,
- reintroduction by homologous recombination of the mutated gene in at least one other thermosensitive (= heat-sensitive) lab strain and verification of the obtaining of fil phenotype showing the monogenicity of the mutation and its non dependency upon the genetic context of the mutated original strain PVD1150.

Specification, paragraph on page 46, lines 1 to 10:

B20

This method has been used with the strain PVD1150 by transformation with the plasmid YCplac33-CYR1 digested by the enzyme *Sna*BI (figure 11). The transformants which grow on minimum medium without uracil (medium SD-URA) have then been selected. These transformants can only grow when there has been a recircularization by the vector, especially coming from an event of double recombination having integrated the missing part of the gene due to the mutated gene *CYR1* in the PVD1150 strain (figure 11). In parallel, the strain M5 has been transformed with the vector YCplac33-CYR1 digested by *Sna*BI, then the transformants have been selected on minimum medium in order to obtain a vector carrying the non mutated gene *CYR1* originated from the control strain M5.

Specification, paragraph on page 48, lines 4 to 10:

B21

The fragment PstI-BamHI of the vector Ycplac33-CYR1^{mut}, fragment which contains the 3' part of the gene *CYR1*^{mut} (including the *fil1* mutation) has been sub-cloned in the vector pUC18. Then the auxotrophy marker *URA3*, originating from vector pJJ242 (Jones and Prakash (1990) Yeast, 6, pp.363-366) has been inserted in this new vector, downstream from the coding part of gene *CYR1*^{mut} in the 3' non coding region, at the level of a unique restriction site (*Sna*BI). The obtained vector, called pUC18-CYR1^{mut}-URA3 is represented in figure 12.

Specification, paragraph on page 55, lines 13 and 14:

B22

The results are collected in table 6-A (evolution of the fermentative activity C1 test) and in figure 13 (stability in frozen doughs maintained at -20°C).

Specification, paragraph on page 55, lines 15 to 22:

B23

A very clear improvement of the stability during freezing was noticed for the 3 mutants AT25, AT26 and AT28 (figure 13) ; under these conditions of test C1, the control strain S47 maintains after 1 month 54 % of its reference gassing power, while the mutants conserve, under the same conditions between 80 and 87% of their reference gassing power. Furthermore and due to the fact that the mutations did not lead to an important penalization of the fermentative ability, all the mutants attain quickly (from 8 to 15 days of conservation at –20°C) levels of fermentative ability higher than those of control S47 (table 6-A).